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# Highly refractory acute myeloid leukemia

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**Summary.** In this study we evaluated 103 patients suffering from acute myeloid leukemia (AML) who did not respond to induction chemotherapy and defined a subgroup of patients with highly refractory disease characterized by a persistence of more than 1 G/L blast cells in the peripheral blood between days 12 and 16 of the first induction cycle. Only seven patients (one female, six males) met these criteria. Their median age was 65 years (range 41–82 years). Four had de novo AML and three secondary AML. Cytogenetic analysis was performed in six patients: complex aberrations were detected in four patients and, unexpectedly, normal karyotypes were found in the other two. Analysis of multidrug-resistance factors revealed high co-expression of P-glycoprotein (P-gp) and lung resistance protein (LRP) in all four patients with highly refractory disease tested a finding in only 6% of patients with refractory disease and 3% of patients who achieved complete remission (CR) of disease. Furthermore, patients with highly refractory AML had substantially higher leukocyte counts than patients with refractory AML or CR, although this was not significant statistically.

Overall, patients with highly refractory AML are characterized by a high incidence of complex cytogenetic aberrations and marked expression of drug transporters.

**Key words:** Leukemia, refractory, multidrug resistance, cytogenetics.

## Introduction

Refractory disease in AML is ill defined. A widely used definition was proposed by Hiddemann et al. [1] and includes nonresponse to first-line treatment. However, the extent of refractoriness among patients nonresponsive to first-line therapy may vary considerably. In some patients peripheral blood counts do not recover after therapy, others achieve normal peripheral blood counts but have persistent bone-marrow blasts > 5%. However, induction chemotherapy usually leads to a clearance of blast cells from the peripheral blood.

In this study we defined a subgroup of patients with highly refractory AML, characterized by a persistence of more than 1 G/L blast cells in the peripheral blood, and aimed to determine the reason for this extreme refractoriness.

## Materials and methods

### *Study design*

In a retrospective study we evaluated 103 patients with AML who did not achieve CR of disease after induction chemotherapy. All patients had received standard induction chemotherapy following published protocols including anthracyclines and cytosine arabinoside [2]. Daily blood counts had been performed between days 12 and 16 of therapy. Twenty-seven patients had white blood counts (WBC) > 1 G/L during this period, and differential blood counts revealed a blast-cell count of > 1 G/L in seven of them. These patients with highly refractory disease were analyzed for risk factors predictive for poor response to chemotherapy, such as cytogenetic aberrations, multidrug resistance, WBC and platelet counts, and compared with other patients with disease refractory to induction therapy but with a peripheral blast cell count below 1 G/L between days 12 and 16 (n = 96), and with patients who had reached CR of disease (n = 342).

### *Cytogenetic analysis*

Cytogenetic analysis was performed on metaphases from short-term bone-marrow cultures (24–72 hours). Methods of cell cultivation, chromosome preparation, and staining using a modified GAG-binding technique have been described elsewhere [3, 4]. Fluorescence in-situ hybridization (FISH) with whole chromosome painting probes, centromere-specific alphoid DNA sequences and single-copy gene probes was carried out in all cases with complex chromosome aberrations [5]. The karyotype was described according to the International System for Human Cytogenetic Nomenclature (ISCN) [6].

### *Detection of FLT3 internal tandem duplication*

In one patient with a normal karyotype (patient number 6) the presence of FLT3 internal tandem duplication was analyzed using PCR [7]. Genomic DNA was amplified using the following primers:

11 F: 5'- GCA ATT TAG GTA TGA AAG CCA GC-3'
12 R: 5'- CTT TCA GCA TTT TGA CGC CAA CC-3.

### *Multidrug resistance*

Expression of P-gp, LRP and MRP1 (multidrug resistance protein 1) was determined immunohistochemically, as described [8]. Briefly, formalin-fixed, paraffin-embedded bone-

**Table 1.** Characteristics of patients with highly refractory AML

| Patient number | Age, years | Sex | de novo/ secondary | FAB subtype | Peripheral blast cell count, days 12–16 |
|---|---|---|---|---|---|
| 1 | 64 | m | de novo | M1 | 2.44 G/L |
| 2 | 82 | m | de novo | M2 | 3.74 G/L |
| 3 | 65 | m | de novo | M2 | 15.56 G/L |
| 4 | 41 | m | de novo | M1 | 1.35 G/L |
| 5 | 61 | m | sec/MDS | – | 19.36 G/L |
| 6 | 72 | m | sec/MDS | M4 | 2.3 G/L |
| 7 | 80 | f | sec/rad | M1 | 74.25 G/L |

*sec* secondary disease; *rad* radiation.

marrow specimens were deparaffinized and endogenous peroxidase activity blocked by incubation in 0.06% $H_2O_2$ for 10 minutes at room temperature. The tissues were preincubated for 20 minutes in normal serum (normal goat serum 1:50; Dako; Glostrup, Denmark) before incubation for two hours with monoclonal antibodies. The following antibodies were used: JSB-1 and MRK-16 for detection of P-gp, LRP-56 for LRP and MRPr1 for MRP1 (all obtained from Alexis, Läufelfingen, Switzerland). Antibody binding was detected with the avidin-biotin-peroxidase method. Bound peroxidase was developed with 3,3'-diaminobenzidine (Dako). The slides were counterstained with Mayer's Hämalaun and mounted with Aquatex (Merck, Darmstadt, Germany). Phosphate-buffered saline was used for all washes. Negative controls were prepared as described above but without monoclonal antibody. Normal human kidney tissue, which is known to overexpress P-gp, LRP, and MRP1, was used as the positive control. Expression of P-gp, LRP and MRP1 was divided into four categories: negative (0% staining blast cells, –), low (≤5% staining blast cells, +), intermediate (6–20% staining blast cells, ++) and high (>20% staining blast cells, +++).

### *Statistical analysis*

The Kruskal-Wallis test was used for calculation of the significance of differences in WBC and platelet counts between patients with highly refractory AML, other refractory AML and CR. The significance of differences between patient subgroups in complex cytogenetics, high pgp-expression, high LRP-expression, high pgp/LRP coexpression and differences of median survival were assessed using the chi-square test. Differences were considered to be significant when the p value was $<0.05$.

## Results

### *Patients' characteristics*

According to the criteria defined above, seven patients (6 males, 1 female) had highly refractory disease. Their median age was 65 years (range 41–82). AML was de novo in four patients. In one patient, AML had developed after radiation therapy for uterine cancer and in two others after myelodysplastic syndrome (Table 1). Three patients received a second cycle of induction therapy, but none of them achieved CR of disease. The median survival was 1.5 months (range 0.7–7.1 months) compared with 3.1 months in patients with refractory disease ($p>0.05$).

### *Prognostic factors*

Prognostic factors analyzed included karyotype, FLT3 internal tandem duplication, multidrug-resistance factors, WBC and platelet counts.

**Table 2.** Multidrug-resistance factors and cytogenetics in highly refractory AML

| Patient | P-gp | LRP | MRP1 | Cytogenetics |
|---|---|---|---|---|
| 1 | nd | nd | nd | 46,XY |
| 2 | nd | nd | nd | 46,XY,der(2)t(2;12)(p25;p11.2),-3,del(5)(q11.2),der(8)(3qter?q21::17?::2?::8p11.2?q24::3?),der(12)t(5;12)(?;p11.2),-17,der(18)t(3;18)(?;q21.3),+22,+mar[23] |
| 3 | +++ | +++ | + | 45, XY, der(2)add(p11.2~13)del(q35),del(5)(q13q33),add(7)(p13),-16[11] |
| 4 | +++ | +++ | – | 48-50,XY,+8,(+8),inv(10)(p11.2q22)c,(-20),+der(20)t(20;21)(q11.2;q11.2)(2-3x),del(21)(q11.2)[28]/46,XY,inv(10)(p11.2q22)c[2] |
| 5 | nd | nd | nd | nd |
| 6 | +++ | +++ | – | 46,XY |
| 7 | +++ | +++ | – | 45,XX,der(3)t(3;17)(q12;?),del(5)(q13q31);-7,+8,r(16),-17[34] |

*nd* not determined.

**Table 3.** Comparison between patients with highly refractory AML (hrAML), other refractory AML (rAML) and CR

| | hrAML | rAML | CR |
|---|---|---|---|
| WBC, G/L, day 0; median (range) | 44.3 (6.0–113.2) | 8.6 (0.6–222.4) | 12.1 (0.2–437.0) |
| WBC, G/L, day14; median (range) | 9.3 (2.3–56.6) | 0.6 (0–20.2) | na |
| PLT, G/L; day 0; median (range) | 49 (8–93) | 47 (2–916) | 41 (3–1110) |
| Complex cytogenetics* | 67% (4/6) | 34% (25/73) | 7% (17/253) |
| Pgp, high expression* | 100% (4/4) | 24% (8/33) | 6% (5/78) |
| LRP, high expression* | 100% (4/4) | 24% (8/33) | 6% (5/78) |
| Pgp/LRP, high co-expression* | 100% (4/4) | 6% (2/33) | 3% (2/78) |
| age; median (range) | 65 (41–82) | 65 (24–86) | 53 (16–89) |

*na* not available; *PLT* platelet count. *Not available from all patients; number of patients positive for the parameter and total number of patients analyzed are given in parenthesis. Differences between patient subgroups in complex cytogenetics, high Pgp-expression, high LRP-expression and high pgp/LRP co-expression are statistically significant ($p < 0.05$).

Cytogenetic analysis was performed in six patients: four had complex aberrations (without a common pattern of chromosomal changes) and two had normal karyotypes (Table 2). In one patient with a normal karyotype analysis for FTL3 internal tandem duplication was negative.

Material for analysis of multidrug-resistance factors was available from four patients. High expression (staining of > 20% of blast cells) of P-gp and LRP was observed in all four patients tested, but no substantial amounts of MRP1 were found (Table 2). The two antibodies used for P-gp gave similar results.

Patients with highly refractory disease had substantially higher WBC than patients with refractory AML or CR, whereas platelet counts were similar in all groups (Table 3). However, differences in WBC were not statistically significant in the Kruskal-Wallis test ($p > 0.05$).

## Discussion

With current chemotherapy, about 70% of patients < 60 years of age with AML and about 50 % of older patients may achieve CR of disease [9, 10]. Prediction of success (or failure) of chemotherapy is important because alternative treatments such as stem cell transplantation might be an option for some patients with refractory disease. Several prognostic factors predictive for poor response to induction therapy, such as age [11], unfavorable karyotype [12–14], presence of drug-resistance proteins [15–21], high WBC [22] and low platelet counts [23], have been described. We studied a group of patients with highly refractory AML, defined by a failure to clear blasts from the peripheral blood at the time of maximum effect of chemotherapy. A cut-off point of > 1 G/L blasts between days 12 and 16 was chosen because at these counts blasts could be identified with a high degree of certainty. Such a high level of resistance to chemotherapy was found in only seven of 103 patients who failed to achieve CR of disease. By studying these patients we hoped to gain greater insight into the mechanisms of refractoriness. Some of the results were not unexpected: patients with highly refractory AML were older than responding patients and WBCs were higher. In addition, a high proportion had complex karyotypic changes, a finding in only 34% of patients with refractory AML and just 7% of patients with CR (Table 3). Surprisingly, two patients had a completely normal karyotype, which is usually related to intermediate prognosis [22]. In an attempt to identify the factors responsible for high refractoriness among patients with normal cytogenetics, we analyzed for the presence of FLT3 internal tandem duplication. This mutation has recently been identified as a prognostic marker in AML [7]. However, in one patient, from whom material was still available, no FLT3 internal tandem duplication was detected.

All four patients tested showed high expression of P-gp and LRP, which underlines the importance of these proteins for drug resistance [15–21]. In contrast to patients with highly refractory disease, only a small subset of those with refractory AML and even fewer with CR were highly positive for P-gp and LRP. High co-expression of P-gp and LRP was found in just four of 111 controls tested (Table 3). Thus, our data confirm that expression of P-gp and LRP, but not MRP1, is highly associated with refractoriness [15–21].

In summary, patients with highly refractory AML are characterized by the presence of a variety of established prognostic factors predictive for poor response to therapy, in particular high co-expression of P-gp and LRP. However, it seems difficult to identify such patients from pretreatment data.

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